# PRIMER EXPRESS

## VERSIONS 1.5 AND 2.0

This tutorial has been prepared by the Computer Support Group of the National Institute of Environmental Health Sciences and is intended for use by employees and contractors with NIEHS. Although descriptions and recommendation appear to be in compliance with the guidelines set forth by Applied Biosystems for the use of Primer Express, neither Applied Biosystems nor its employees are responsible for the contents of this tutorial.

The appendices in this tutorial were taken from the installer disk for version 1.0 of Primer Express and from a tutorial provided by Applied Biosystems and are reproduced with their permission.

# Introduction

Primer Express is a primer design program from Applied Biosystems that also allows for an easy design of compatible TaqMan probes, for use in real-time PCR or end-point PCR analyses. It can also be used for the design of primers alone when the user expects to analyze samples with SYBRGreen only. It will, therefore, be assumed that anyone taking this class is already familiar with the concepts behind real-time PCR and the relative advantages and limitations of using TaqMan probes or SYBRGreen. Primer Express exists in a version 1.5 for use on a Macintosh platform and a version 2.0 for use on a PC platform. Only very minor differences exists between the two versions and these should be noted during the class.

By the end of this class, the users should be able to design primers and probes for their sequences of interest. They should also be able to identify and use the different documents (search routines) available within Primer Express and the annotation tools that can be used to limit their searches. Finally, they should be able to understand how the default parameters, which define the searches, might be adjusted for more difficult sequences.

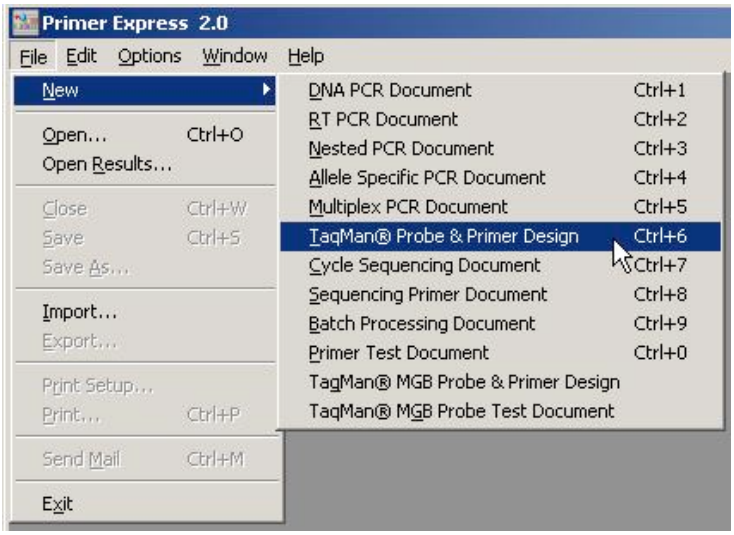
# Table of Contents

# A Quick Start

## Initiating a new Primer/Probe search for Tamra-quenched probes.

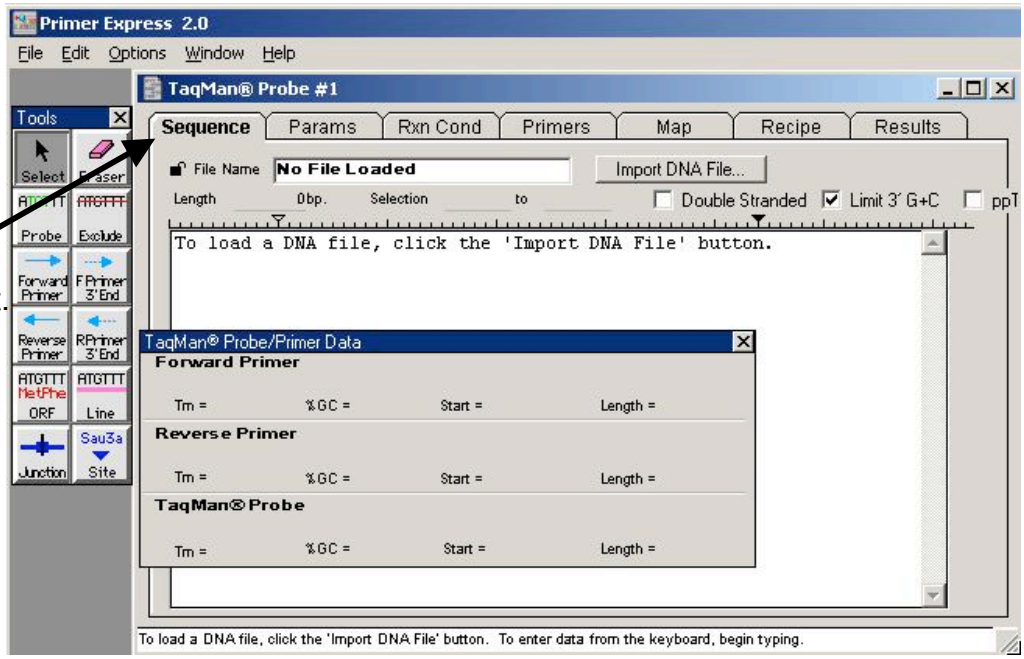
### Activate TaqMan® Probe & Primer Design

1. After opening Primer Express, From the **File** menu choose **New**
2. Slide to the right and from the cascading menu choose **TaqMan® Probe & Primer Design**.



This will open up the **TaqMan® Probe** search screen at the **Sequence** tab.

**Sequence** tab opens by default.



## Introduce a target sequence

| **Note**: Although not officially documented, longer sequences (>3,000?) have been observed to yield less-than-optimal primer/probe sets or (>10,000?) no sets, at all. **Sequences of 300-1,000 bases typically yield abundant, optimal sets.** |
|---|

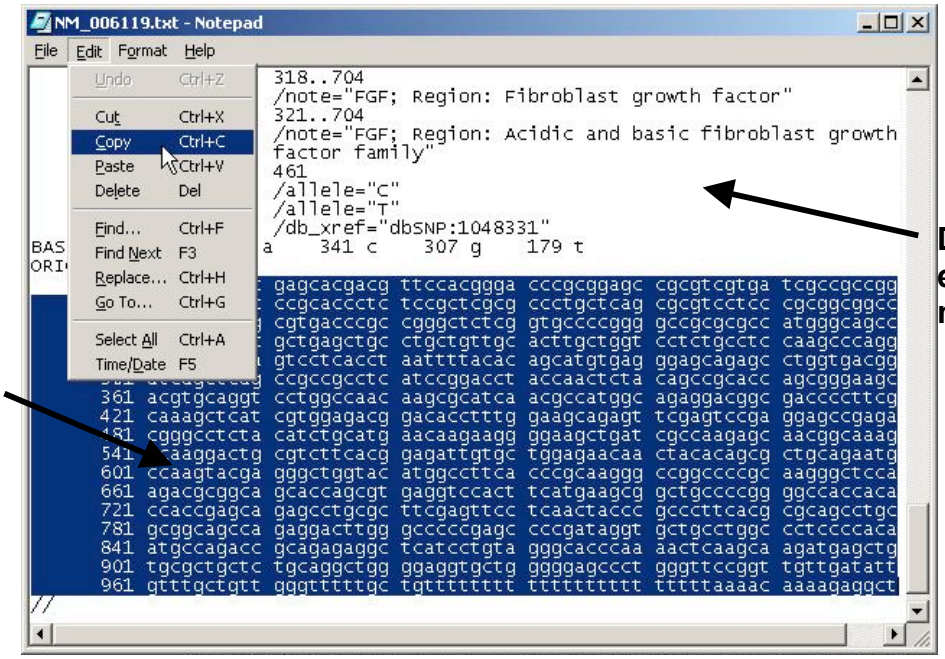
There are two methods for bringing a target sequence into the TaqMan® probe screen:

- Copying and pasting a sequence from another document.
- By importing it.

### Copy and paste

To **copy** the sequence:

1. Select the nucleotide sequence from a Word or Text document.
   See the example of the Notepad file NM_006119 below.
2. Highlight the nucleotide sequence
3. Copy it onto the computer's clipboard. From the **Edit** menu choose **Copy.**
   Keyboard shortcuts: **Control(Ctrl)+C** in Windows
   **Command(⌘)+C** in Macintosh.



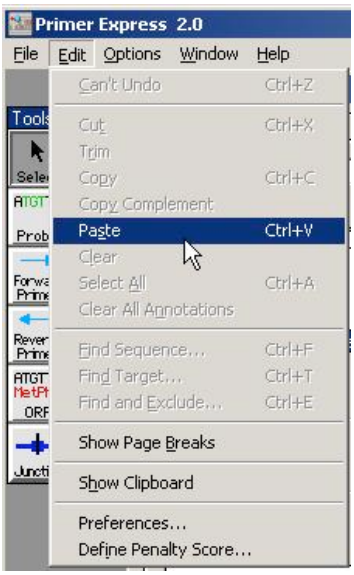

**Highlight nucleotide sequence.**

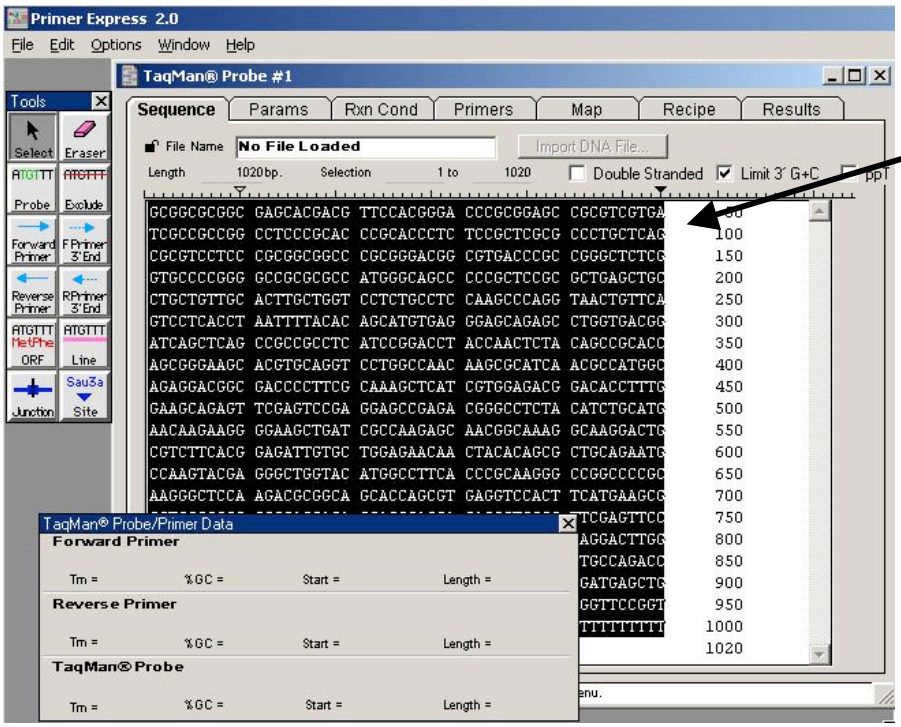
**Do not include extra text or numbers.**

**Note:** The numbers and/or annotations are okay but do not include extraneous information, such as the description of the sequence.

To **paste** the sequence:

1. Return to the Primer Express window.
2. Select the Sequence tab (if it is no longer active).
3. Paste the sequence. From the **Edit** menu choose **Paste.**
   Keyboard shortcuts: **Control(Ctrl)+V** in Win.
   **Command(⌘)+V** in Mac.




**The pasted sequence will appear in the Sequence screen. It automatically replaces any text already in the screen.**

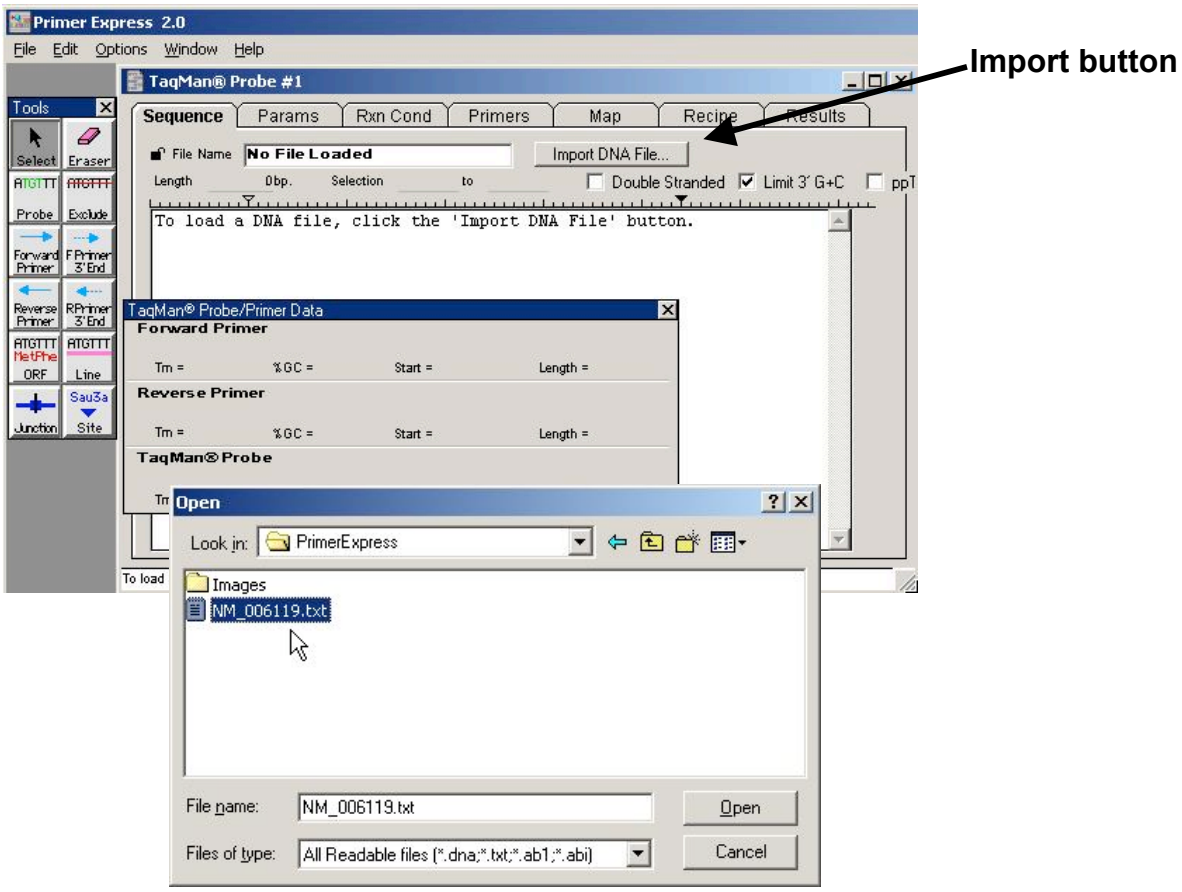
**Note:** The annotations may have changed.

**Import the nucleotide sequence**

Instead of copy and paste, a sequence can be Imported into Primer Express:

> **Note:** The sequence must be in a tab-delimited text format.

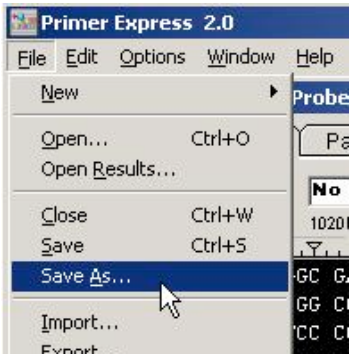
1. Click the **Import DNA File** button near the top of the **Sequence** page.
2. The Open file window will appear.
3. Navigate to the folder that contains your sequence.
4. Select the file and choose **Open**




> **Note: Before importing**, remove all extraneous information from the file so it contains only the sequence. The numbers and/or annotations are okay. If the text is in a **GenBank, EMBL, FASTA, GCG, or ASCII** format, descriptive information will automatically be removed.

## Save a sequence

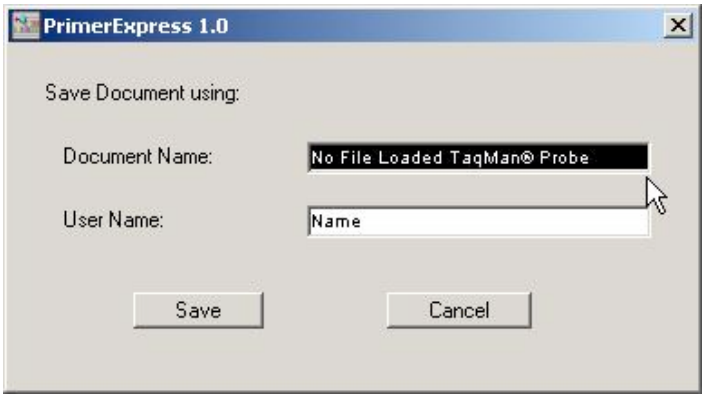
The sequence can be saved in the **Primer Express Archive Folder.** (This is the default folder and cannot be changed.)




1. From the **File** menu choose **SaveAs.**

The **Save Document** window appears.

2. In the **Document Name** field type the name for this sequence
3. The **User Name** field will specify the Primer Express Archive Folder.
4. Select the **Save** button




The subsequent file will also specify that it was created for evaluation in the TaqMan Probe and Primer Design document.

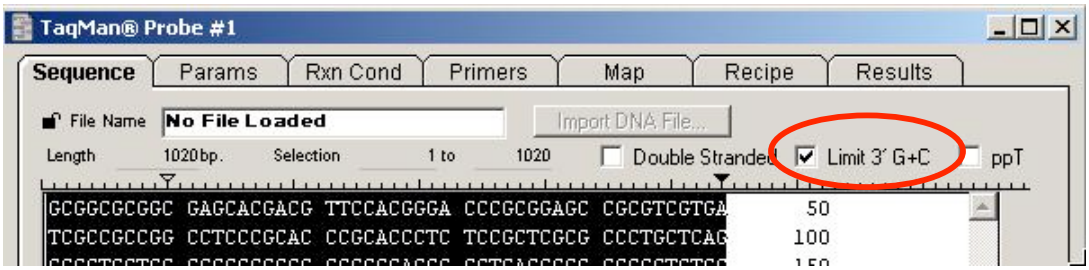
## Start the Primer/Probe Search

### Generate acceptable primer/probe sets for the sequence

1. Select the **Sequence** tab (if it is not already active).
2. Verify that the box labeled **Limit 3' G+C**, at the top of the **Sequence** page, **is** checked.

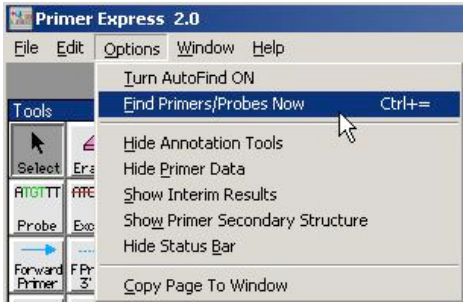
| | |
|---|---|
| **Mac Users:** | Version 1.5 is **unchecked** by default |
| **Win Users:** | Version 2.0 is **checked** by default. |

3. Check the **Limit 3' G+C** box if it is not checked.




4. From the **Options** menu choose **Find Primers/Probes Now**

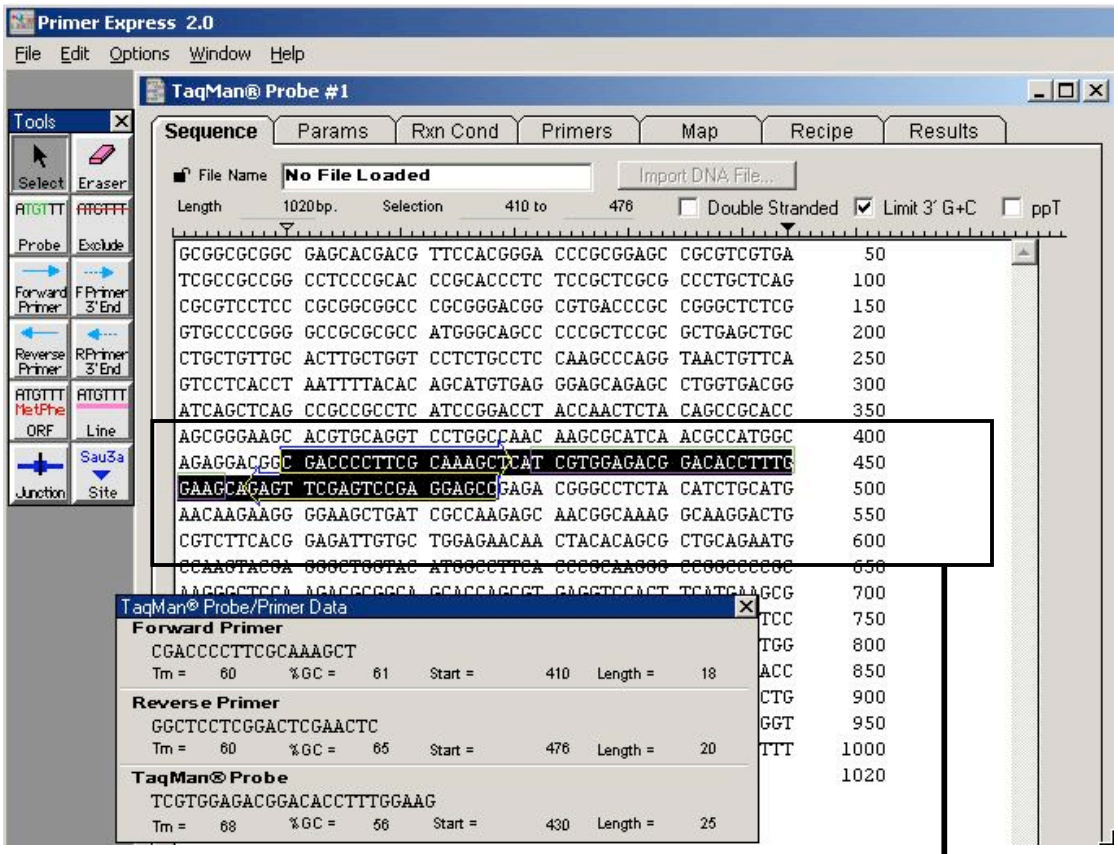
   Keyboard shortcuts: **Control(Ctrl)+=** in Win.
   **Command(⌘)+=** in Mac.



## View the generated acceptable primer/probe sets.

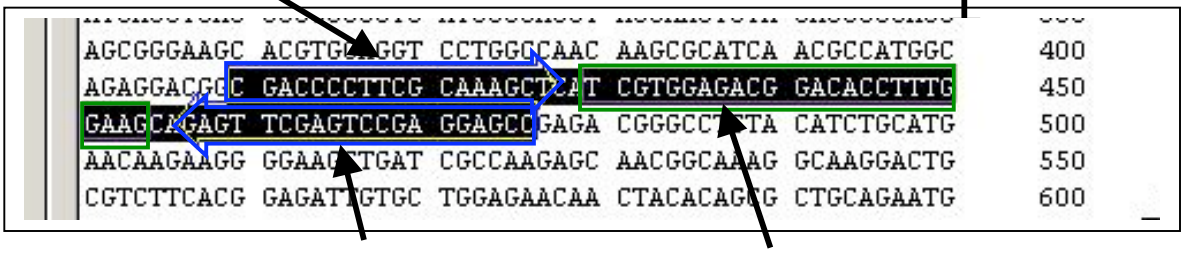
The generated list contains up to **200 primer/probe sets**. Only **one** of the suggested sets is displayed on the **Sequence** screen.

There are **two(2)** ways to immediately view the selected primer/probe set on the **Sequence** screen.




**1st** view on **Sequence** screen

The **Forward Primer** is indicated by the **Blue** arrow pointing to the **right**.



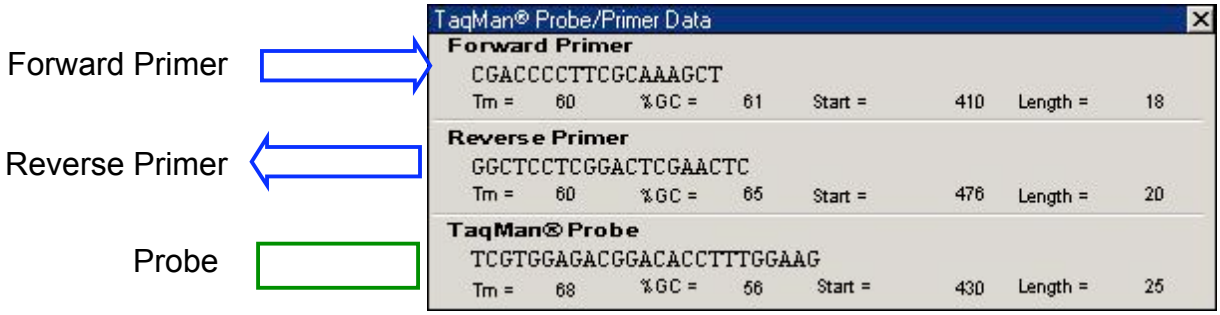

The complement of the **Reverse Primer** is indicated by the **Blue** arrow pointing to the **left**.

The **Probe** is indicated by the **Green** box.

**Note**: The reverse strand can be displayed by checking the **Double Stranded** box at the top of the Sequence page.

## 2nd view on the **Sequence** screen

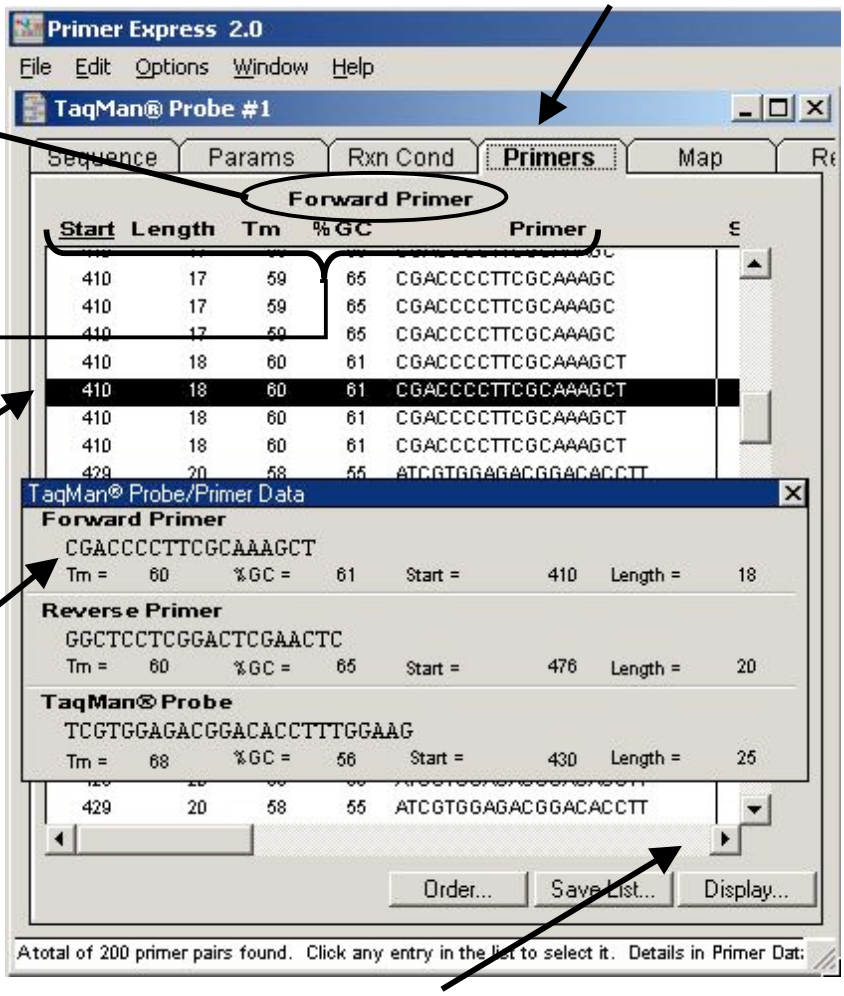
**TaqMan® Probe/Primer Data** window located elsewhere on the screen. This window displays the same primer/probe set highlighted on the screen and includes some relevant parameters.




**Note:** The Theory of Operation by which these sets were selected is explained in more detail in **Appendix A.**

## View the entire list of primer/probe sets

The entire list of primer/probe sets is under the **Primers** tab. Up to 200 sets are available.




The screen may be too narrow to view more than the **Forward Primer** members of the sets. Scroll to the right to view Reverse Primers and Probes.

Click on **labels** to **sort** by that column.

The selected primer/probe set on the **Sequence** screen is highlighted here.

**TaqMan® Probe/Primer Data** window is still visible and displays highlighted set. For more information on this window, see page 9.

Scroll to the right to view **Reverse Primers and Probes.**

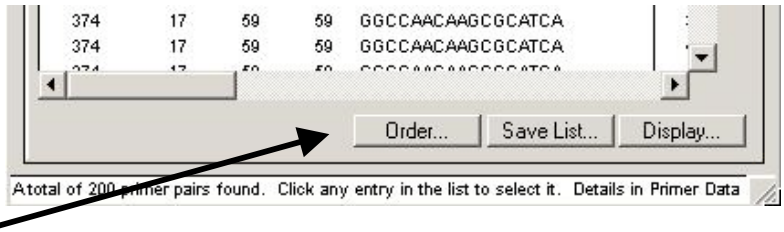
To select a different Primer or Probe click on it.

The TaqMan® Probe/Primer Data window will change to reflect the new choice.
The Sequence Screen will also change to reflect the new choice

**Note:** Any of the sets in this list will have passed the screening parameters (filters). Don't consider the first set chosen to be the only or best option.

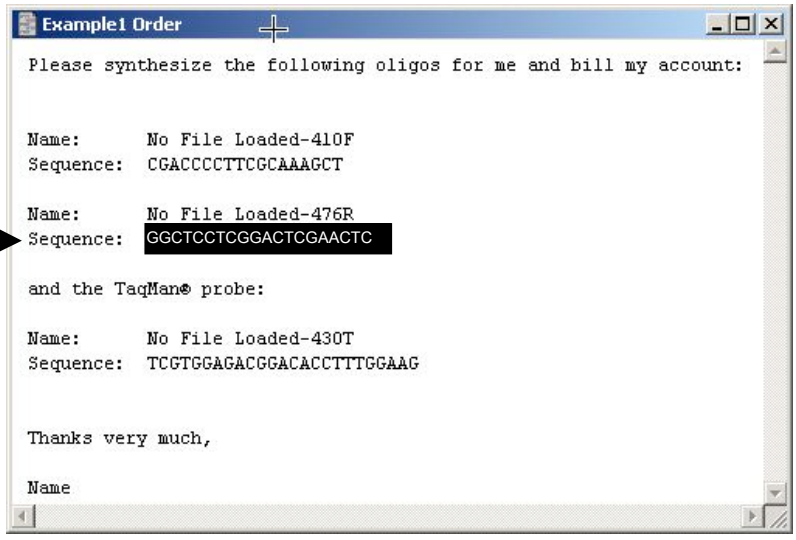
## Get information for ordering a sequence

### The Order Button



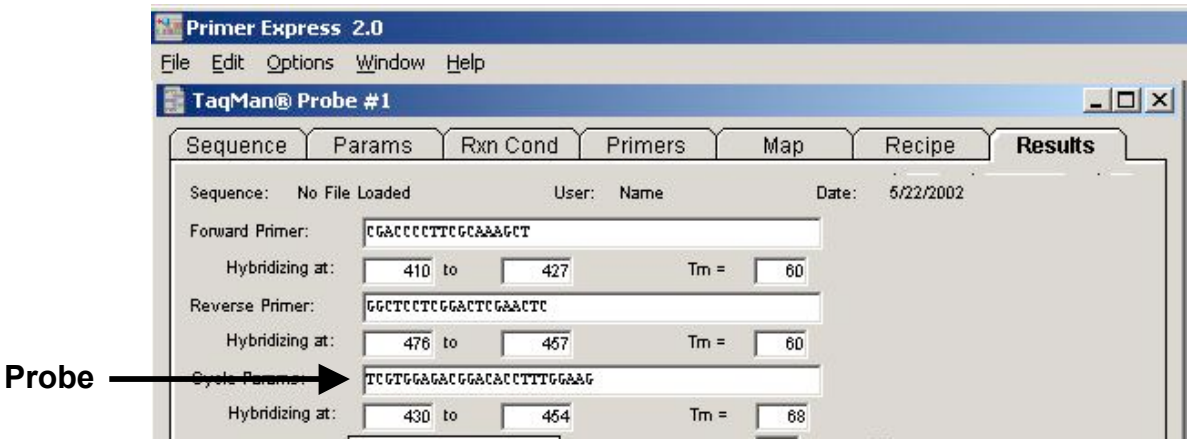

The **Order…** button at the bottom of the **Primers** page will display a text file describing the highlighted primers and probe from which the sequences can be copied and pasted into another text document.

**Copy** and **Paste** any sequence



### Results tab

This screen lists all the members of the highlighted set with some more detail than the TaqMan® Probe/Primer Data window.




**Results** tab is useful because the primer and probe sequences can be copied from this page and pasted into other documents for records or for ordering them from other suppliers.

| |
|---|
| Note: The probe is incorrectly labeled **Cycle Params:**.<br>Also the data on the bottom half of the screen has been cut out of this picture because it is not useful for the present version of the software. |

## Design guidelines

Because most of the previously recommended guidelines (**Appendix B**) are now incorporated into the software, only one significant adjustment must be made.

## Proposed probe composition

A. By default, the probe displayed is on the forward strand.

B. The complement probe will have the same annealing temperature and would fit most of the same filters.

C. The Probe with more C's than G's will perform better.

D. Count the nucleotides to determine which probe has more C's.

E. Only choose the complement if it does not have a G at the 5' end.

## Restricting a search

**Facts:**

- Only 200 sets can be listed under the Primers tab.
- Possibly many more passed all of the default filters but could not be displayed in this abbreviated report.
- You may want to limit the search to distinguish mRNA from gDNA, to distinguish a particular gene from a family of similar genes, or to include genes from multiple species, for example.

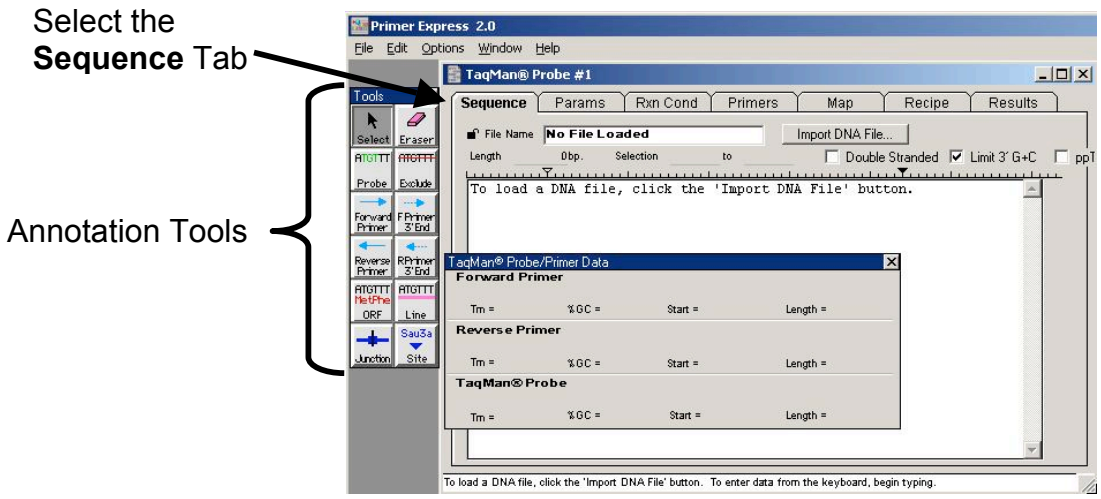
**Conclusion:**

You may want to add custom restrictions to the filters to better refine your search.

## Annotation tools

1. Select the **Sequence** tab.

2. The **Eight(8) Tool buttons** are visible to the left of the **TaqMan®** **Probe** window.




**Note:** A more complete description of the tools is included in **Appendix B** of this document.

**Using the tools**

1. Press the button of the desired tool
2. Highlight the corresponding site or sequence.

**Useful tools for TaqMan searches are:**


The Junction button can be used to specify a location across which a probe must be located. For example, placing a probe across an intron junction will prevent genomic DNA from being amplified in conjunction with mRNA.

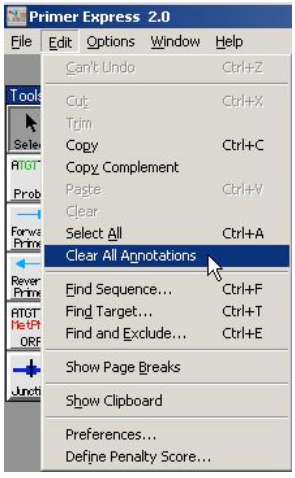

The Site button can similarly be used to identify a particular location (including an intron junction) but does not impose the probe restriction. A short description can be entered at the designated site.


The Exclude button can be used to designate a region of the sequence which the user does not want to be included in the search for primers or probes.


The Line button can similarly identify a sequence of interest without imposing any restriction on the primer/probe search.


The Forward Primer, Reverse Primer, and Probe buttons can be used to specify any one of the corresponding oligonucleotides. Typically, they might be used to "lock in" one of the components from the initial search, that is at a desirable location, to generate a list of sets with other components that would be compatible with it.


The eraser can be used to remove any of the previously placed annotations.




Note: If there are several annotations and the user wants to remove all of them, from the **Edit** menu choose **Clear All Annotations**.

## Opening some of the Filters

If the initial search does not return any acceptable primer/probe sets, it may instead be necessary to alter or ease some of the restrictions on their design.

**WARNING:** The default parameters were designed to provide the optimal designs and should not be altered without considering that doing so will be likely to yield less-than-optimal primers and/or probes.

### Limit 3' G+C" box

Deselect the **Limit 3' G+C" box** located at the top right of the **Sequence** page

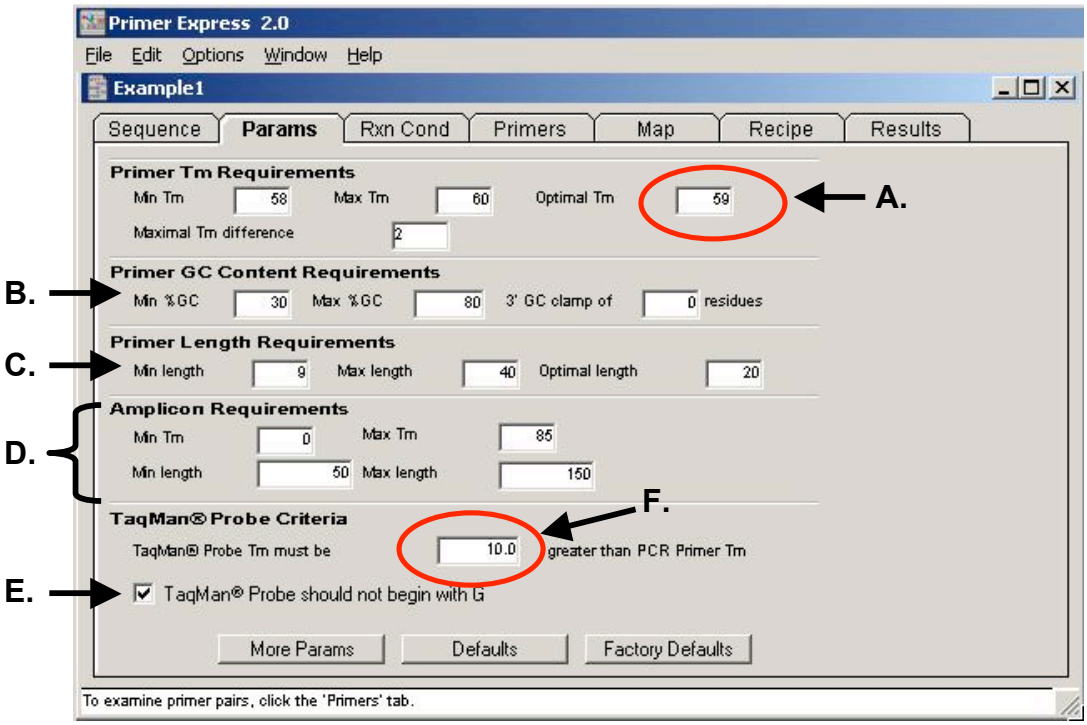
**Note:** Performing a search without having this limitation is likely to yield some primers with more than two G's or C's in the final five bases at the 3' end of the sequence.

**Outcome:** Three **G's** or **C's** may still be acceptable, particularly if they are not sequential and/or they are not at the very end of the sequence.

**Avoid:** Three or more **G's** or **C's** may facilitate formation of a **GC Clamp**, which could interfere with the optimal function of the primers, by facilitating primer-dimer formation, and should be avoided.

**The Params tab.**

Many of the filter adjustments can be found under the **Params** tab




A. **Annealing temperature ($T_m$)**: Although Applied Biosystems **strongly** recommends that the annealing temperature ($T_m$) of primers be left at **59°** to maintain standard conditions, this page is where the user could change this parameter.

B. **GC requirements %GC:** The **GC** content of the primers could be changed although it is **not** recommended.

C. **Length requirements:** The length parameters could be adjusted although it is **not** recommended.

D. **Amplicon Length:** The Amplicon length can be changed.

**Max Length:** The **150 bp** upper limit is intended to ensure that the PCR will have close to 100% efficiency. If this limit were to be increased, it should be anticipated that the PCR efficiency will be **decreased**.

**Min Length:** While it is unlikely that an amplicon could be designed with less than 50 bp, this lower limit could also be increased (for example to create a larger amplicon for visualization on a gel) up to the Max Length.

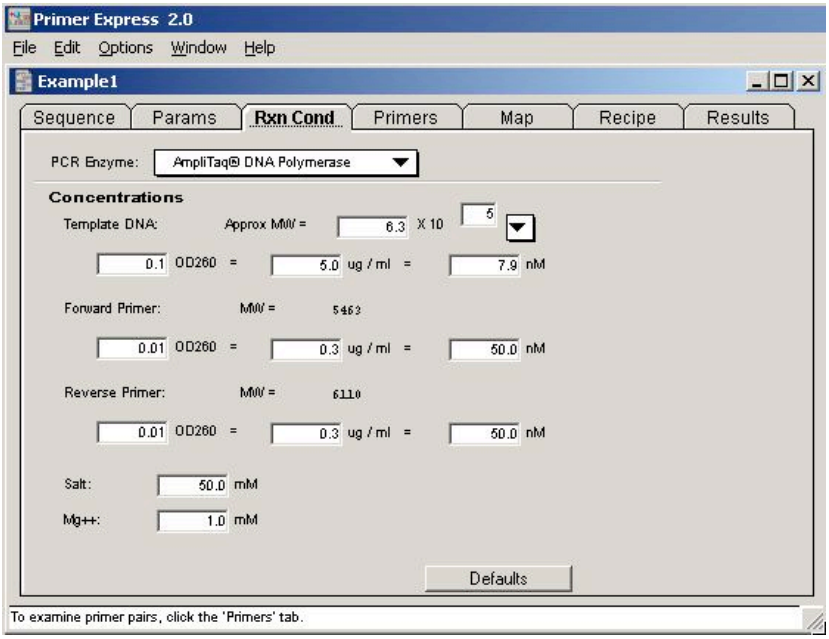
E. **Probe beginning:** Probes should **not** be designed with a **G** at their **5'** end.

F. **Probe Annealing Temperature:** The annealing temperature of the probe at **10° above** the annealing temperature of the primers should **not** be changed **except** when designing probes for **allele discrimination**.

## Rxn Cond and Recipe tabs

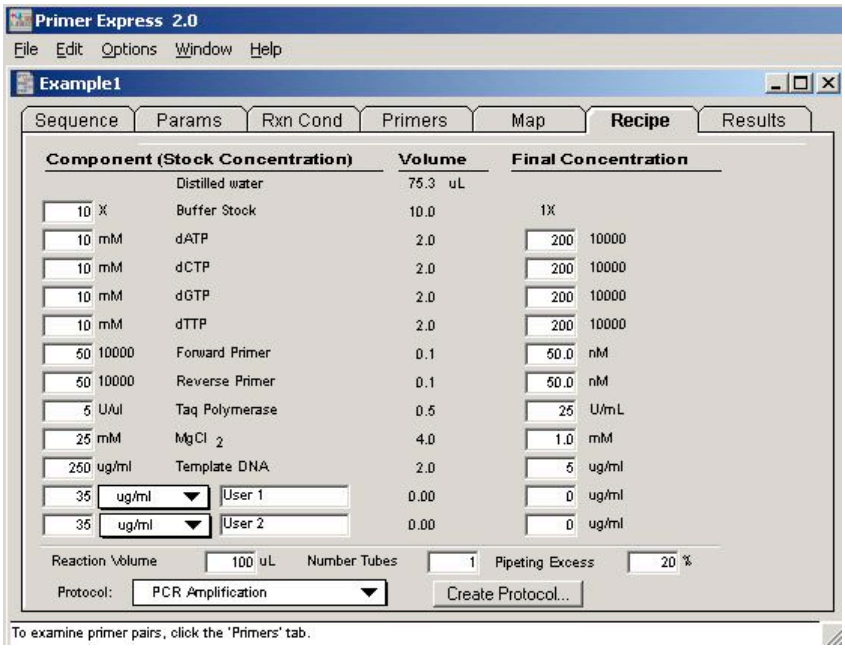
Although the parameters listed under the **Rxn Cond** and the **Recipe** tabs may appear to be inconsistent with Applied Biosystems mixes and standard reaction conditions, the user **SHOULD NOT CHANGE** any of them.

### Rxn Cond tab




Although they may appear to be incorrect, some of these values may actually be modified and used in some of the calculations used by the Primer Express software.

## Recipe tab



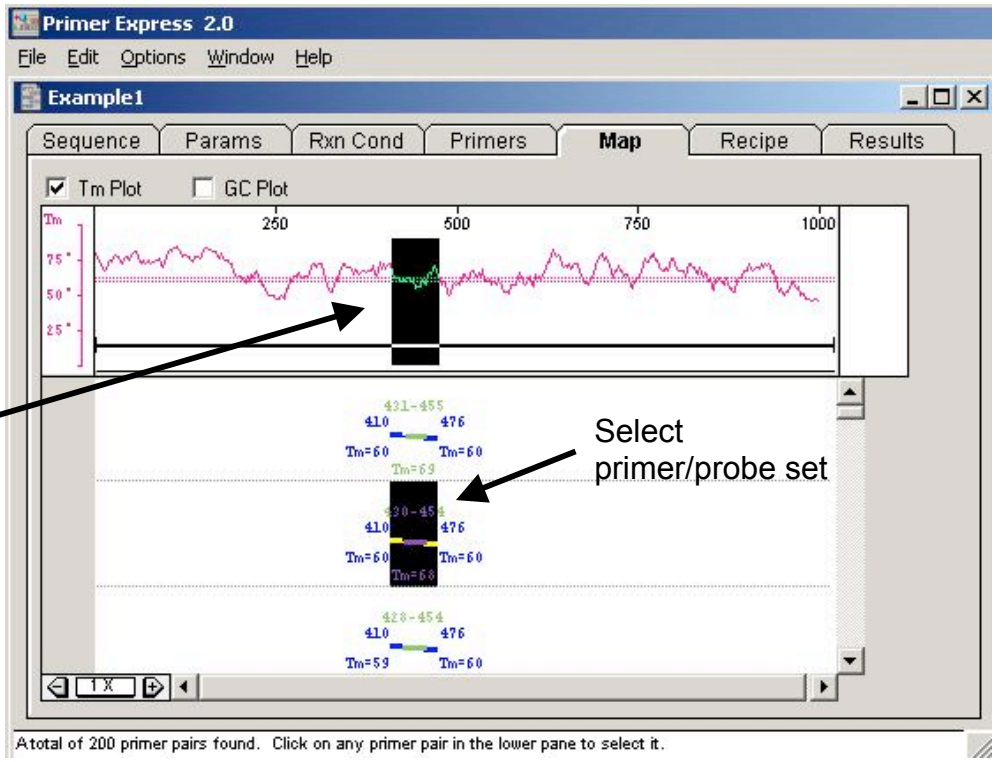

**WARNING:** Changing **Rxn Cond** or **Recipe** tabs can result in improperly designed primer/probe sets. These pages will be eliminated in future versions of the software to avoid confusion.

### The Map tab

The display under the **Map** tab illustrates the **relative position** of the primers and probes listed under the **Primers** tab.

**Compare a primer/probe to:**

1. The position of any annotations that were put on the sequence
2. The $T_m$ estimates along the sequence.




This feature can be used to scan through the sets to locate those that may be in a region of interest or to identify a region that is likely to contain acceptable sets, based on the calculated $T_m$ values, for a more limited search.

## Documents

To locate other Documents, from the **FILE** menu choose **NEW**. Although a more complete description of the documents is listed in Appendix D, the majority of the documents currently listed under this menu are of little utility for a TaqMan® Primer/Probe search.

| **NOTE:** | Most document choices found on the **FILE**, **NEW** menu are **not** useful for a TaqMan® Primer/Probe search. |
|---|---|

## Useful Documents

### TaqMan® MGB Probe & Primer Design

This document is necessary for designing **MGB probes**.

It incorporates the influence on the Probe **Tm** that results from the **MGB "tail"**.

| **Note:** The complement may not have the same Tm. |
|---|

### DNA PCR

Users who anticipate using only **SYBRGreen®** are still encouraged to design complete primer/probe sets. This prepares them for any future decisions to use **TaqMan® probes** instead of SYBRGreen.

Using probe restrictions may occasionally prevent the search from returning any acceptable primers. In this case, the **DNA PCR Document** can be used to find **only primers** that pass the default filters.

1. Removes the probe restrictions from the search
2. Uses the same restrictions that have been set.
3. The **Junction** tool is located between chosen primers rather than within a probe.

**Primer Test**

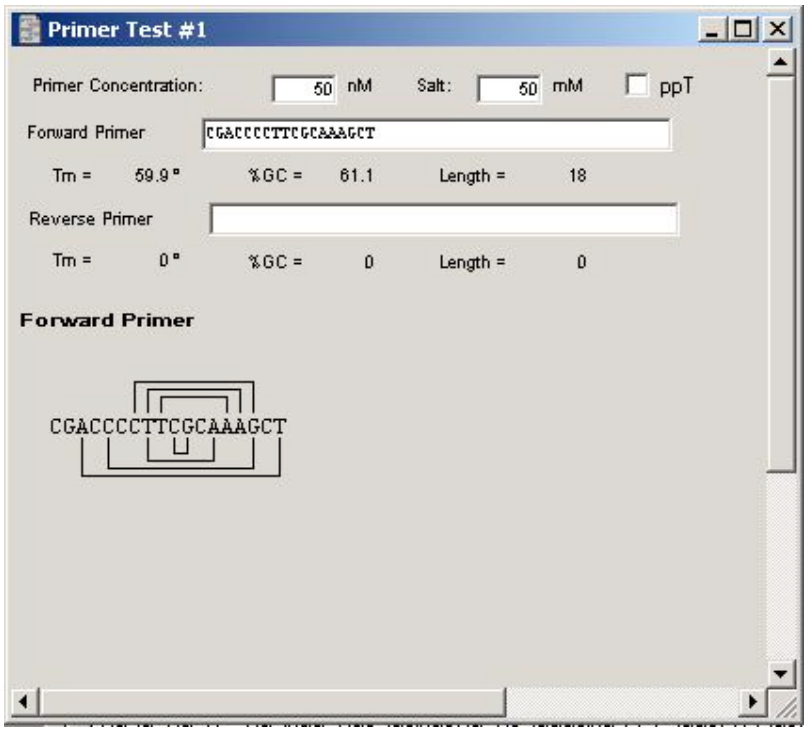
Test potential sequences for **primer** or **(non-MGB) probe** consideration by copying and pasting the sequence of interest into the **Primer Test Document**.

To use the **Primer Test** document:

1. From the **FILE** menu choose **NEW**
2. Slide over and choose **PRIMER TEST**
3. Select the **SEQUENCE** tab
4. Highlight the Forward or Reverse Primer you wish to test
5. From the **EDIT** menu choose **COPY** (or **COPY COMPLEMENT**)
6. Place cursor in the Forward or Reverse Primer field
7. From the **EDIT** menu choose **PASTE**

Primer Test document shows:

A. Tm
B. GC content
C. Length of the sequence entered.
D. Potential self-complementarity.
E. Potential primer-dimer formation.




These could be severe enough to have eliminated the sequence from consideration by one of the filters in the search program.

| **Note:** | This information could be used to adjust default filters |
|---|---|

The **Primer Test Document** can also be used to examine potential probes by copying and pasting. To examine the probe complements:

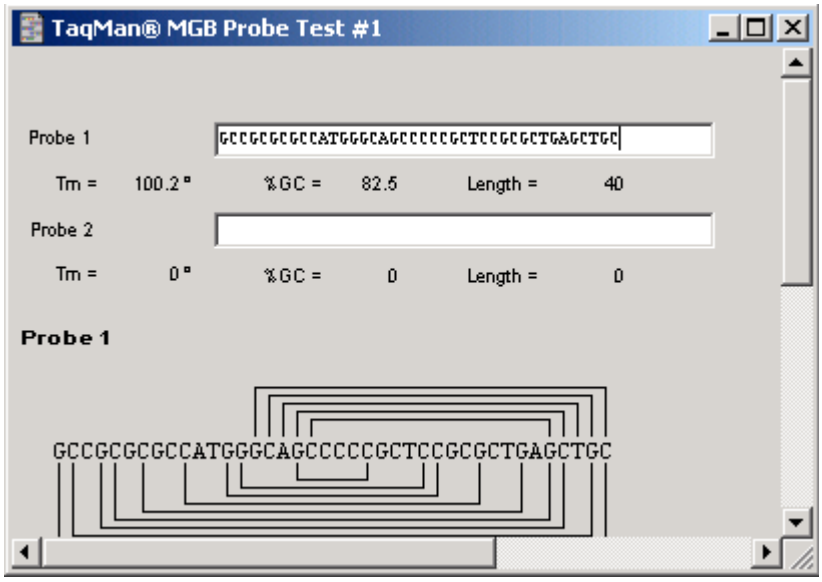
1. Select the sequence of interest
2. From the **EDIT** menu choose **Copy** or **Copy Complement** from the Sequence listing (for the forward or reverse sequence, respectively) and **Paste** into the Primer Test Document.

## TaqMan® MGB Probe Test

The **TaqMan® MGB Probe Test Document** incorporates the effect of the **MGB** on the **Tm** of the probe.

To use the **MGB Probe Test** document:

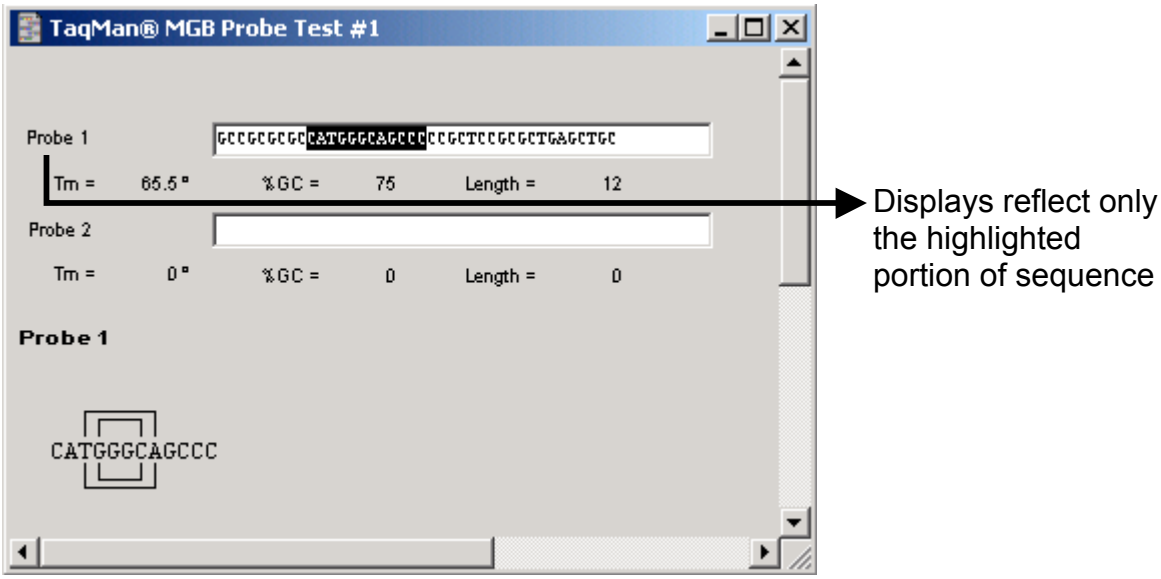
1. From the **FILE** menu choose **NEW**
2. Slide over and choose **TaqMan® MGB Probe Test Document**
3. Select the **SEQUENCE** tab from the **MGB Probe & Primer Design Document**
4. Highlight the probe you wish to test
5. From the **EDIT** menu choose **COPY** (or **COPY COMPLEMENT**)
6. Place cursor in the Probe 1 or Probe 2 field
7. From the **EDIT** menu choose **PASTE**



**Test a sequence**

A sequence in either of these fields can be randomly tested.

1. Highlight a portion of it.
2. The Tm and other characteristics displayed correspond to the highlighted portion.




**Use an acceptable sequence**

If an acceptable sequence is thus identified, Remove the remaining sequence. From the **EDIT** menu choose **TRIM**.

| | |
|---|---|
| **Note:** | That the complement sequence probably won't have the same **Tm** because the effect of the **MGB** is influenced by the nearest nucleotides. |

8. The chosen sequence can be "locked" into a search on the **MGB Probe & Primer Design Document,** using the Probe annotation tool, as described on page 15.

# Appendix A: Summary of Design Guidelines

## TaqMan Probe:

1. Keep the G-C content in the 30-80% range.
2. Avoid runs of an identical nucleotide. This is especially true for guanine, where runs of four or more Gs should be avoided.
3. Do not put Gs on the 5' end.
4. Select the strand that gives the probe more Cs than Gs.
5. For single-probe assays, Tm should be 68-70°C when using Primer Express software.

## Primers:

1. Choose the primers after the probe.
2. Design the primers as close as possible to the probe without overlapping the probe.
3. Keep the G-C content in the 30-80% range.
4. Avoid runs of an identical nucleotide. This is especially true for guanine, where runs of four our more Gs should be avoided.
5. When using Primer Express software, the Tm should be 58-60°C.
6. The five nucleotides at the 3' end should have no more than two G and/or C bases.

# Appendix B: Annotation Tools

The use of some of the Annotation Tools can affect what primer pairs are returned by the program:

## Target Tool:

Selecting a region with the Target Tool tells Primer Express to require that any primer pair returned would result in the region being amplified. If a target has been selected by the user, Primer Express tests each candidate primer pair to determine if the target region is amplified. The number of such primer pairs is shown in Interim Results as Number Passing Required Test.

## Exclude Tool:

Selecting a region with the Exclude Tool tells Primer Express not to return any primer pairs that would amplify any of the sequence marked as excluded. If a region has been marked as excluded by the user, Primer Express tests each candidate primer to determine if any excluded region is amplified. All primer pairs that avoid excluded regions are tested further, and the number of such primer pairs is shown in Interim Results as Number Passing Avoid Ignores Test.

## Primer Tools:

The Forward and Reverse Primer Tools allow the user to specify one or both primers by dragging through the sequence. Primers specified by the user do not need to meet the criteria specified in the Params page. Users may also specify just the 3' most residue of one or both primers using the F Primer 3' End Tool or the R Primer 3' End Tool. Primer Express does test primers against the criteria in the Params page when this tool is used, and, in addition, only examines subsequences ending with the specified residue.

## Junction Tool:

The Junction Tool can be used to require Primer Express to return only those primer pairs where at least one primer of the pair crosses at least one juction in the template. The usual use for this is to create primers that will only amplify mRNA (or cDNA made from it) and not genomic DNA by marking the positions of the exon junctions with this tool.

## Appendix C: Documents

The same basic steps are used for finding primers in each of the document types supported by Primer Express, with variations based on the particular of the document type:

### RT PCR:

Finds primers exactly as does DNA PCR, the only difference being that RT PCR documents look for and remove intron features in imported DNA sequences, and mark their location with a Junction annotation.

### Nested PCR:

Follows all the steps described above, but additionally examines each acceptable primer pair to find those that nest within each other. All such sets, each made up of an internal primer pair and an external primer pair, are returned to the user.

### Allele Specific PCR:

Unlike any of the other Primer Express document classes, this document imports alignments of sequences. The user can then mark which sequences in the alignment should be amplified by any returned primers and which should not be amplified. Primer Express then determines which residue positions discriminate between sequences in the amplified set and those in the not-amplified set. A normal primer pair seach then takes place, but each primer pair found is evaluated to determine if it contains a discriminatory residue. The position of the discriminatory residue in the primer is a criterion that the user can set using **Discriminatory residues must be within the last x residues from 3' end** in the **Primer Composition Requirements.** Only those primer pairs that contain discriminatory residues within the specific region are returned to the user.

### Multiplex PCR:

The goal of Multiplex is to find many sets of primers that can be used in the same PCR reaction to amplify many different sequences simultaneously. The user can specify any number of targets within a sequence using the Target Tools, or, import multiple sequences, with the assumption that each of these sequences should be amplified by one primer pair. For multiplex PCR to be accomplished the primer pairs must meet all the usual criteria, but in addition, the $T_m$ values for all of the primers in the reaction must be fairly close, and no primer dimer reactions can occur between any of the primers in the reaction. In addition, the amplified products of the various PCR reactions must differ from each other enough in length that they can be distinguished by electrophoresis. Primer Express finds multiplex sets that match these criteria by first finding all acceptable

primer pairs for each of the targets, then testing every combination and returning those sets that meet the criteria. A special criterion on primers in multiplex is the **Last four positions contain** value, which, by requiring that all the primers have lots of C's but no G's (or lots of G's but no C's, etc.) at their 3' ends, essentially prevents the possibility of primer dimer. Other criteria include the **Maximal $T_m$ difference between sets** value, which guarantees that all primer pairs in the multiplex have similar $T_m$s, and the **Min spacing** value, found in the **Amplicon requirements,** which ensures that the amplicon lengths are different enough to be distinguishable.

**TaqMan:**

The TaqMan document returns sets composed of a primer pair and a TaqMan probe. Primer Express finds these by first finding all acceptable primer pairs, using the standard criteria, and then finding TaqMan probes that would hybridize between the primer pairs. The user can set two criteria for the TaqMan probes: The minimum amount by which the TaqMan probe $T_m$ exceeds the $T_m$ of the primer pairs, and whether TaqMan probes should be restricted from begining with G. Primer Express tests candidate TaqMan probes using essentially the same methods used for candidate primers to determine $T_m$, to search for significant internal secondary structure, and to determine whether the candidate probe has significant sequence similarity to any other region of the sequence. Only those candidate probes that pass these tests are returned to the user as TaqMan sets.

**Sequencing Primers:**

Primer Express uses the same criteria to evaluate candidate sequencing primers that it used to evaluate PCR primers. In addition, however, the user can specify **Primer Position Requirements** to make certain that all primers returned are a specified distance from the end towards which they prime.